REMARKS

Reconsideration and allowance of the above-identified application is respectfully requested.

Please consider this paper a Request for Continued Prosecution. Please also consider this paper a petition for a 1-month extension of time. The required fees are attached. If the fees are missing or deficient, please charge our deposit account 500687 under order No. 95773.

Claims 1-7, 9-16 and 19-42 are pending in the application.

Applicants' counsel thanks Examiners Tiffany M. Gough and Ruth Davis for the courtesy extended during the Interview of 20 April 2007. The above claim amendments and arguments are commensurate with the discussions during the Interview. The differences between the cited prior art and the claimed invention was discussed during the interview, which are pointed out again below. The attached Rule 132 Declaration includes the experimental evidence discussed during the interview.

Claims 1, 19 and 20 have been amended to recite removing the activated carbon. Support for this amendment can be found in the originally filed application, including at page 5, lines 20-21. Basis for new claims 21- 42 can be found in the originally filed application. Basis for claims 24, 25, 34 and 35 can be found by combining the teaching on page 4, lines 15-16 of the specification, which teaches that the diluted enzyme has at least the same level of activity as the undiluted enzyme, with the minimum dilution factor of 3 times (otherwise known as 1/3 of the original quantity), i.e. 200%. No new matter has been added.

Applicants reserve the right to pursue the subject matter of the original claims, including other purifying agents besides activated carbon, in a continuation application.

On page 2 of the pending Office Action, the Examiner refused to consider the non-patent literature publications submitted on 3/11/2004. Applicants respectfully submit that copies of the two publications by Kerkhoff and Lilljedahl were submitted. Applicants submit herewith copies of both publications. The relevance of these two

publications is discussed on page 9, first paragraph, and page 13, last paragraph to page 14, second paragraph of Applicants' Response filed 19 October 2006. Applicants submit that the 3/11/2004 Information Disclosure Statement fully complies with the rules and full consideration of the publications is respectfully requested.

Attached herewith are copies of the non-patent publications discussed below and a FORM/PTO/SB08A. Applicants request that the Examiner fully consider the non-patent publications and initial the attached FORM/PTO/SB08A.

The rejection of claims 1-7 and 9-16 under 35 U.S.C. § 112, second paragraph, on page 2 of the pending Final Office Action is respectfully traversed. The words "to a substrate susceptible to said enzyme" were added to provide antecedent for "substrate" for claim 20. Examples of suitable substrates are disclosed on page 4, lines 26-29, which includes polysaccharide products. Applicants submit that the claims fully comply with Section 112. Accordingly, withdrawal of the Section 112 rejection is respectfully requested.

The rejection of claims 1-3, 6, 7 10, 19 and 20 under 35 U.S.C. § 102(b) as being anticipated by Bailey (U.S. Patent No. 4,204,041) is respectfully traversed. The claimed invention is not anticipated by Bailey for the following reasons.

As discussed during the interview, Bailey discloses use of an enzyme that is irreversibly attached to the activated carbon and wherein the latter bound material must be present in the medium where and when the enzyme-catalyzed reaction takes place. Thus, Bailey does not teach forming a diluted enzyme solution, but rather a support containing bound enzyme.

In contrast, the present invention does not involve an enzyme-bound to agent system. The present invention forms a diluted enzyme solution having enhanced activity. The claims have been amended to recite removing the activated carbon as set forth above to further distinguish the claimed invention from Bailey.

Bailey does not teach or suggest the claimed steps. Accordingly, withdrawal of the Section 102 rejection is respectfully requested.

The rejection of claims 1-6, 9, 19 and 20 under 35 U.S.C. § 102(b) as being

anticipated by Lausten et al (U.S. Publication No. 2002/0020668) is respectfully traversed. The claimed invention is not anticipated by Lausten for the following reasons.

As discussed during the interview, Lausten discloses dilution of an enzyme broth with water at an upper or maximum 1:1 ratio.

In contrast, the claimed invention requires far more dilution, at least by a factor of 3 times.

Furthermore, Lausten teaches diluting an enzyme broth, which contains cells, and then contacting the enzyme broth containing cells to carbon Picatif FGV 120.

In contrast, the claimed invention contacts an enzyme broth or solution, in which the cells have been filtered out first, with activated carbon.

Lausten does not disclosed the claimed steps. Accordingly, withdrawal of the Section 102 rejection is respectfully requested.

The Examiner's states on page 5 of the Office Action, that:

> Although the above references do not specifically state the enhancement of the enzyme activity, the method of treating an enzyme solution with a purifying agent, activated carbon, is the same, and further it is known in the art that activated [carbon] is a purifying agent of enzymes and by purifying a substance one is further enhancing its intrinsic properties/activities, therefore, the enhancement of activity must be an inherent property of mixing such solutions together.

Attached herewith is a Rule 132 Declaration which demonstrates that contact with activated carbon alone does not result in an enhancement of activity. Only the claimed steps of dilution, removal of cells if present, and then contact with activated carbon results in a surprising enhancement of activity, which can be on the order of 1000% or by a magnitude of 10. This unexpected increase in activity is not taught or suggested by the cited prior art.

Applicants respectfully submit that this statement is incorrect. The Examiner contends that treatment of enzymes with activated carbon, or indeed, any purification step, should automatically lead to an enhancement of activity. However, Lausten is

silent on this matter, and Bailey recorded a decrease in activity after enzymes were "mixed" with activated carbon. It is generally known throughout the art that purification leads to a decrease in enzyme activity. Enhancement of enzyme activity is not an inevitable outcome of purification as is apparently believed by the Examiner. To support Applicants' position, the following evidence is presented.

Every basic course in biochemistry and proteomics teaches protein purification, and the basic tenet is that purification is a challenge, fraught with many adverse outcomes. While it may be possible to overcome some of these challenges, some proteins cannot be effectively isolated in an active form.

The Cornell lab manual for BIOBM330 discusses strategies for protein purification. (http://instruct1.cit.cornell.edu/Courses/biobm330/protlab/Strategy.html) The manual specifically states:

> Because of structural and functional differences between proteins, an ideal sequence of steps for one protein will, quite possibly, be unsuccessful for another. A knowledge of the theoretical basis of each procedure will allow the researcher to choose an initial sequence of techniques with which to attempt any given purification. However, the development of an optimised protocol involves considerable trial-and-error experimentation to assess the potential of each step

The manual also specifically states:

> Note that the yield of enzyme after a particular purification procedure may be low not because the procedure is failing to purify that protein, but because it is causing some inactivation of the enzyme.

Based on these statements, it is apparent that the procedure is not automatic – the fact that a procedure works provides no guarantee that it will work for another enzyme. Furthermore, even maintenance of activity is difficult, and enhancement of activity is not automatic.

Instructional materials for the MATC Biotechnology program in Madison, WI discuss methods and goals for protein purification.

http://matcmadison.edu/biotech/resources/proteins/labManual/chapter_1.htm

They state:

> The ideal purification strategy has the following goals: maximum recovery of the target protein; minimal loss of biological activity; and maximum removal of contaminating proteins, as well as low cost...

Note that one of the goals is to minimize loss of activity – enhancement of activity is difficult, although specific activity may increase, depending upon one's ability to concentrate the desired protein.

There is significant supporting evidence in the research literature as well, in spite of the fact that the tendency would be to publish successful separation methods, and not report the failures. For example, Wingfield et al. (Eur. J Biochem. 180.23-32 (1989) studied the purification of a wild-type and recombinant aminopeptidase, and noted significant loss of activity during purification, likely due to loss of essential metal ions. On page 28, they state: "The enzyme, fully active before the separation step, was completely inactive after the separation..."

Mackay et al. (Fundamental and Applied Toxicology, vol 30, pp23 – 30, 1996) studied a protein with neuropathy target esterase activity, and noted (page 26):

> This suggests there was an appreciable deactivation of the enzyme during purification. This trend was seen throughout the purification process; large amounts of protein without NTE activity were removed from the active fraction with no appreciable increase in NTE specific activity.

Additional citations from the literature are included in attached Appendix.

These examples, among many others, demonstrate that the purification/separation method does not automatically lead to an enhancement of activity. Indeed, these authors report loss of activity during purification, which is indeed a common problem, and a challenge to be overcome, as noted in the instructional materials at Cornell and MATC. Thus, the examiner's claim that enhancement of enzyme activity is an inherent outcome of purification is wholly inconsistent with the science of protein purification.

The Examiner contends that processing with activated carbon, as a well-established purifying agent, should automatically enhance enzyme activity. Again, the

literature demonstrates that such an outcome is not automatic. For example, Pimenov et al. (Sep. Sci. Technol., 36(15), 3385-3394, 2001) studied microbial growth and survival following contact with activated carbon. The authors discovered that contact with activated carbon had a significant adverse impact on microbial growth and enzyme activity:

> "The experiments provided conclusive evidence that adhesion of microbes onto the activated carbon fiber directly influences bacterial metabolism". In this case, "The esterase metabolic activities of the adsorbed microorganisms were measured and were found to diminish rapidly and cease completely in less than 24 hours."

In an undated report, Hydamaka et al. from the Food Science Department at the University of Manitoba evaluated the use of activated carbon to control color problems and enzymatic browning in recycled food process waters. The observed that with a proper loading of activated carbon, the activity of the phenolase enzyme responsible for browning could be reduced to zero.

In conclusion, Applicants contend that the above evidence and references, in addition to those additional references included in Appendix, clearly demonstrate that processing with activated carbon alone does not lead to an enhancement in enzyme activity.

The rejection of claims 12, 13 and 15-17 under 35 U.S.C. § 103(a) as being unpatentable over Shenoy et al (J. of Bioscience, vol. 7, 1985) in view of http://www.ap-lab.com/circular_dichroism.htm ("AP-Labs") and Lausten is respectfully traversed. The claimed invention is not taught or suggested by the theoretical combination of Shenoy, AP-Labs, and Lausten for the following reasons.

Before addressing the Examiner's arguments, Applicants submit that the experimental evidence of record and presented in the attached Rule 132 Declaration demonstrate the unexpected advantages of the claimed invention compared to the cited references.

The experimental evidence demonstrates that merely contacting the enzyme solution with activated carbon does not result in an increase in enzyme activity. See

the graphs in the Rule 132 Declaration, center bars, which are the same height as the left most bars representing the original enzyme solution. In contrast, when the enzyme solution is diluted with an aqueous solution, cells are removed when present, and then contacted with activated carbon, an astonishing 10 fold (1000%) increase in enzyme activity is obtained. See the graphs in the Rule 132 Declaration, right most bars of each three bar set. If the enzyme activity did not increase 10 fold, the right most bars would be 1/10 the height of the left most bars for each set of three bars. For this reason alone the Section 103 rejection should be withdrawn.

The Examiner argues that Shenoy teaches "[t]he specific activity of the purified enzymes was three times higher than that of the original non-purified glucoamylase (see p. 400)." However, p. 400 of Shenoy in fact teaches that "[t]he specific activity of the purified A candidus glucoamylase was 436 which is three time higher than that of the **parent strain** already reported (Manjunath and Raghavendra Rao, 1979)." [Emphasis added.] Thus, Shenoy does **not** teach that purification results in an activity that is three times higher than the original non-purified glucoamylase, but rather compared to a parent strain.

The experimental evidence in the attached Rule 132 Declaration demonstrates that merely contacting an enzyme with activated carbon does not result in an increase in enzyme activity. The general knowledge in the art, demonstrated by the many articles submitted herewith and discussed in the Attachment, also confirms that contact with activated carbon does not result in an increase in enzyme activity, as discussed below. Applicants discovered that the enzyme solution must be filtered to remove cells if present, diluted with an aqueous solution, and then contacted with activated carbon to provide the unexpected increase in enzyme activity. Thus, Shenoy does not teach the unexpected advantages of the present invention.

The Examiner admits that "Shenoy does not teach lower CD spectrum ranges such as those claimed by applicant nor alpha-amylase." However, the Examiner contends that "a change in the spectral range appears to be an inherent property of purification", citing the AP-Labs website as evidence.

In fact, in contrast, the goal of purification is to avoid a structural (and spectral) change, as demonstrated by the following statement in instructional materials from Cornell University: "Purification procedures attempt to maintain the protein in native form. Although some proteins can be re-natured, most cannot!"

The Examiner cites information found at http://www.ap-lab.com/circular_dichroism.htm, stating that this website teaches that "any change in structure of proteins will affect the CD spectral range". This is indeed true, but Applicants respectfully submit that the Examiner's subsequent conclusion that "a change in spectral range appears to be an inherent property of purification" is incorrect. As noted in a multitude of other texts and references on protein purification discussed below, the goal is to preserve structure, because this is the most effective way to preserve activity. Changes in structure are, in most instances, correlated with loss of activity, due to chemical or thermal denaturation. Tools such as circular dichroism and x-ray crystallography are thus used to examine protein structure, ostensibly to determine if purification has had an adverse impact on protein structure.

In an authoritative review on the use of circular dichroism, Kelly and Price (Curr. Protein and Peptide Sci., 1, 349-384, 2000) state (page 373) that CD is used to "address the question of the retention of native structure on extraction, purification and crystallisation." Thus, CD is used after the fact, to ensure that the native structure has not been altered by extraction, purification, or crystallization. Additional supporting references are included in the attached Appendix.

The claimed process is unique in that it leads to a change in protein structure, which is believed, without being bound by any theory, to be due to catalytic modification of the protein, that enhances the specific activity of the protein, unlike a traditional purification process that simply acts to concentrate a protein. In a traditional purification process, there is an inevitable reduction in (active) protein, but an even greater reduction in volume, resulting in a product solution that is more concentrated in the desired protein. In the present process, Applicants have effected an increase in the volume of enzyme solution, i.e. dilution, while increasing the enzyme activity, in some

cases even maintaining the baseline activity of the original (already purified) enzyme solution. This multi-fold enhancement in enzyme activity per unit mass of protein is believed to be due to catalytic transformation of the protein, changing its structure, and leading to a change in its spectral properties as measured by CD and by UV.

Consequently, the Examiner's statement that "a change in the spectral range appears to be an inherent property of purification" is inconsistent with widely-held knowledge about the process of protein purification, since an ideal purification procedure would preserve protein structure, and avoid a change in spectral properties.

The Examiner argues that "Lausten teach[es] the use of activated carbon in a fermentation broth, particularly with enzymes such as amylases and cellulases, to remove soluble impurities by purification improving the quality of a product." However, as discussed above, Lausten teaches contacting an unfiltered enzyme broth containing cells to the carbon, which is different from the claimed process. Present claims 1, 2, 4-7, 9-16, 19 and 20 recite "raw enzyme solution." This term is defined in the present application on page 2, lines 26-36 as having the cells removed. New claims 21-40 specifically recite removing the cells if present. Thus, in the claimed process, the cells are removed before the enzyme solution is contacted with the activated carbon. Furthermore, in the claimed process, the enzyme solution is diluted with an aqueous solution by at least 3 times before being contacted with the activated carbon.

The combination of Shenoy, AP-Labs, and Lausten does not teach or suggest the claimed process of contacting a diluted enzyme solution to activated carbon, in which the cells have been removed.

The Examiner states that "Applicant absolutely claims a specific CD Spectra in claims 12-15, thus applicant arguments are not supported by the claims" at page 8, last line, through page 9, top. [Emphasis added.] This statement is not understood by Applicants. Reference to claims 11 to 15 listed below shows changes in the defined spectra as highlighted by Applicants' underlining in the claims.

> 11. A method as defined in claim 1 wherein said enzyme solution of enhanced activity has a spectrum selected from Far UV (CD) and UV

visible spectra *distinct* from said raw enzyme solution.

12. A method as defined in claim 11 wherein said enzyme solution of enhanced activity shows a relative absorbance intensity *lower* than said raw enzyme solution, in the CD spectral range of 205-230 nm.

13. A method as defined in claim 11 wherein said enzyme is alpha-amylase and said enzyme solution of enhanced activity has a Far UV (CD) spectrum minimum ellipticity *shifted* by at least 1 nm, from the raw enzyme solution, in the range between 205-230 nm.

14. A method as defined in claim 1 wherein said enzyme solution of enhanced activity has a UV-visible spectrum maximum peak at least 30 nm *lower* than said raw enzyme solution.

15. A method as defined in claim 1 wherein said enzyme is alpha-amylase and said enzyme solution of enhanced activity has a maximum spectral absorption peak over the range 340 to 360 nm.

It is clear that these claims define a *change* in spectral properties, not an absolute spectrum as stated by the Examiner.

In view of the many differences between the claimed invention and theoretical combination of Shenoy, AP-Labs and Lausten, and the unexpected advantages of the claimed invention, withdrawal of the Section 103 rejection is respectfully requested.

The rejection of claims 1-3, 5, 10, 11, 14, 19 and 20 under 35 U.S.C. § 103(a) as being unpatentable over Aikat in view of Bailey or Lausten is respectfully traversed. The claimed invention is not taught or suggested by the theoretical combination of Aikat and Bailey or Lausten for the following reasons.

Before addressing the Examiner's arguments, Applicants submit that the experimental evidence of record and presented in the attached Rule 132 Declaration demonstrate the unexpected advantages of the claimed invention compared to the cited references.

The experimental evidence demonstrates that merely contacting the enzyme solution with activated carbon does not result in an increase in enzyme activity. See the graphs in the Rule 132 Declaration, center bars, which are the same height as the

left most bars representing the original enzyme solution. In contrast, when the enzyme solution is diluted with an aqueous solution, cells are removed when present, and then contacted with activated carbon, an astonishing 10 fold (1000%) increase in enzyme activity is obtained. See the graphs in the Rule 132 Declaration, right most bars of each three bar set. If the enzyme activity did not increase 10 fold, the right most bars would be 1/10 the height of the left most bars for each set of three bars. For this reason alone the Section 103 rejection should be withdrawn.

The Examiner has acknowledged that Aikat does not teach diluting the raw enzyme solution prior to treatment with a activated carbon. Applicants respectfully submit that one of ordinary skill in the art would not have been motivated to dilute an enzyme solution prior to purification with activated carbon with a reasonable expectation for successfully enhancing the intrinsic activity of such solution, because the art does not teach such success when using the claimed enzyme solution or any other enzyme solution and activated carbon.

Furthermore, Bailey and Lausten do not teach the claimed steps of diluting to a level of at least 3 times, removing cells if present, contacting with activated carbon to form an enzyme solution having enhanced activity, and removing the activated carbon. As discussed above, Bailey teaches forming a bound enzyme. Lausten teaches diluting an enzyme broth containing cells only to a level of 2 times (100%) and contacting the solution containing cells to carbon. None of the cited references, alone or in any combination, teach the claimed steps of dilution at least 3 times (200%), removing cells if present, and then contacting the diluted solution with activated carbon. For this reason alone, the Section 103 rejection should be withdrawn.

The cited references do not teach or suggest the unexpected results of the claimed invention. The key to the results of Aikat is their electrophoresis results. The images of the crude protein and the charcoal-treated proteins show that the smaller proteins were almost completely removed by the activated carbon. This is clearly a quintessential case of protein purification, not dilution according to the present invention.

By comparison, the claimed process results in an extra band when glucoamylase is treated, and the smaller proteins are more abundant when amylase is treated (shown as A, A', B, B' in Figure 3). The observations are consistent with a structural change in the native protein, but not consistent with the simple purification described by Aikat. It can therefore be concluded that Aikat have performed a simple purification – small proteins, debris, and extracellular nucleic acids have been removed by adsorption and/or size exclusion, while leaving the treated protein unaffected. In contrast, the claimed process leads to transformation of the enzyme and dilution accompanied by enhanced activity.

There is nothing within Lausten to suggest an increase in activity arising from treatment with activated carbon and the Examiner acknowledges that Laustsen does not "specifically state the enhancement of enzyme activity", although "the method of treating an enzyme solution with a purifying agent, activated carbon, is the same." The Examiner goes on to state that it is well known that activated carbon is a purifying agent for enzymes, and by purifying a substance, one is further enhancing its intrinsic properties/activities. The Examiner ultimately concludes that "the enhancement of activity must be an inherent property of mixing two such solutions together". This conclusion is, in fact, not justifiable for the many reasons discussed above regarding the state of the art, which teaches that activated carbon does not provide enhanced activity.

Indeed, if the Examiner's conclusion was correct, Bailey should have seen an increase in activity when they contacted enzyme with activated carbon during immobilization, but they did not – in fact, in their patent, they explicitly state that there was no benefit observed when some types of activated carbon were used in their process. If there was an automatic benefit associated with combining activated carbon and enzyme, as the Examiner implies, then Bailey should have consistently seen such a benefit, but they did not. Furthermore, their study published in Biotechnology and Bioengineering demonstrated that the intrinsic activity of the enzymes decreased as a consequence of contacting/attachment with activated carbon. Bailey defines an

immobilization factor (IF) that denotes the overall change in enzyme activity during the process. The values of IF in Table I range from 0.208 to 0.381, meaning that activity was lost after contact with/immobilization to activated carbon, even on the smallest activated carbon particles. Similarly, IF values for glucose oxidase (Table III) range from 0.023 to 0.085, meaning that at least 90% of the original enzyme activity is lost after contact with activated carbon. Furthermore, Bailey use their data to specifically conclude that there has been "a reduction in intrinsic activity of glucose oxidase due to immobilization" (page 1934, first three lines of paragraph 4). This is in direct conflict with the Examiner's conclusion that "enhancement of activity must be an inherent property of mixing two such solutions together".

To achieve the stabilizing benefits outlined by Bailey, the activated carbon must remain in the system; otherwise, it cannot remove the peroxide the enzyme is continuously exposed to during the reaction catalyzed by the enzyme. This is distinct from Applicants' process, where the enzyme is contacted with activated carbon, but then the activated carbon is removed. Thus, the benefits from Applicants' process are not due to activated carbon's role as a stabilizing agent, because, in Applicants' process, only the treated enzymes are present in the reaction medium that contains the inactivating agents described by Bailey. As Bailey so effectively demonstrated, their process hinges on the ability of activated carbon to remove peroxide from solution; if the activated carbon is not present (or its surface is blocked, as in the case of glutaraldehyde coupling), no benefit is conferred. Since, in Applicants' process, the enzyme is separated from the activated carbon before it is used, the activated carbon is unable to provide the benefits outlined by Bailey. Consequently, there is nothing taught by Bailey that would lead one to anticipate that treatment of an enzyme by an appropriate amount of activated carbon could lead to an increase in the activity of the enzyme or a change in the structure of the enzyme, particularly if the enzyme is ultimately used independently of the activated carbon itself.

In view of the many differences between the claimed invention and the theoretical combination of cited references, and the unexpected advantages of the

claimed invention, withdrawal of the Section 103 rejection is respectfully requested.

In view of all of the objections and rejections of record having been addressed, Applicants submit that the present application is in condition for allowance and Notice to that effect is respectfully, requested.

Respectfully submitted,
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ATTACHMENT - Additional Supporting References

A. References related to protein purification:

1) Lendenmen and Spain (J. Bacteriol., pp6227 – 6232, 1996) studied 2-Aminophenol 1,6-Dioxygenase purified from *Pseudomonas pseudoalcaligenes* JS45, and observed (p6229):

"Dialysis against 50 mM MOPS (pH 7.3) containing ethanol (10% [vol/vol]) for 24 h abolished 95% of the activity of 2-aminophenol 1,6-dioxygenase. Incubation of the dialyzed enzyme with ferrous iron and ascorbate could restore only 10% of the initial activity. Loss of activity during dialysis is another common phenomenon among extradiol dioxygenases"

2) Chen et al. (Plant Physiol. 118: 199–207, 1998) attempted to purify a recombinant form of D-Ribulose-5-Phosphate 3-Epimerase, and observed (p 203):

"Although these contaminants were effectively removed by Mono-Q chromatography, which resolved two peaks (I and II) of epimerase, the specific activity was somewhat compromised because of a modest loss of units during the preceding dialysis."

3) G. Walsh, Proteins: Biochemistry and Biotechnology, Wiley, West Sussex, England (2002)

The book contains a large number of comments about protein purification, protein structure and protein activity in Chapter 3. The following is a brief selection

"Any influence that alters the protein's native structure will likely influence its biological activity (in the vast majority of cases its biological activity will be decreased or abolished)" (page 156)

"Loss of biological activity can occur during
- *a) protein recovery from its producer source*
- *b) during the protein purification procedure or*
- *c) subsequent to purification, during protein storage"* (page 156)

"Proteolysis can be minimized by undertaking the initial stages of protein purification as quickly as possible" (page 159)

"Proteins vary widely in their susceptibility to any given inactivating influence, and their relative stability or lability in any given circumstance ultimately depends on their structure" (page 156)

"Vigorous agitation is amongst the most common physical influence that can lead to

loss of protein activity." and *"Agitation is usually a feature of cellular disruption, but can also occur during the actual protein purification process"* (page 160)

4) A. Sadana, Bioseparation of Proteins, Academic Press, San Diego (1998)

To emphasize the significance of the problem, Sadana has five chapters discussing the loss of protein activity during purification. These are:

Chapter 4 (pp 101–134) Interfacial Protein Adsorption and Inactivation During Bioseparation

Chapter 5 (pp 135–176) Protein Inactivations during Chromatographic Methods of Separation

Chapter 6 (pp177–212) Protein Inactivation during Novel Bioseparation Techniques

Chapter 7 (pp 213–259) Adsorption Influence on Bioseparation and Inactivation

Chapter 9 (pp 287–312) Protein Refolding and Inactivation during Bioseparation

The preface (page x) states "*some denaturation during bioseparation is unavoidable*"

Page (xi): *"Comparisons of protein inactivation during different bioseparation processes provide valuable information for workers in different areas who are interested in bioseparations*"

Page 2: "*In general, protein purification techniques should be simple, easily scalable, continuous, low cost; and, of course, should not inactivate the protein.*"

Various passages in chapter 1 discuss the inevitable link between separation and loss of activity resulting from changes in structure caused by the purification process. Various sections in chapter 5 link activity loss to chromatographic separation techniques. Some examples:

Page 135: *"A major problem in the effective utilization of chromatographic techniques is the extent of protein denaturation…"*

Page 136: "*When competing hydrophobic and coulombic forces on the column are greater than those maintaining protein structure, denaturation can occur. Mobile phases can also cause denaturation.*"

From chapter 6 (page 178): *"Most proteins must be folded into a specific three-dimensional*

conformation to express their biological activities and specificity, which further complicates the process of separating and purifying them. Also, each separation process is specific for a particular process due to the rather unique characteristics of each bioproduct separated. This often tests the knowledge of engineers, chemists and others to the very limit." (On the basis of the last two sentences, it is apparent why any particular separation outcome is NOT obvious)

5) M. Ladisch, R Willson, C Painton, S. Builder (eds) Protein Purification: From Molecular Mechanisms to Large Scale Processes, ACS Symposium Series 427 (1990)

Article by P. Bailon and S. Roy, "Recovery of Recombinant Proteins by Immunoaffinity Chromatography", pp 150-167. Page 163 states:

"The identity, quality and biological potency of a protein are dependent on its primary, secondary, and tertiary structure." "Full biologic activity usually confirms that at least the tertiary structure of the biologically relevant (interacting) domains is correct."

Page 163 also states *"Unnatural disturbances in the secondary or tertiary structure of the intact molecule can render the molecule partially or completely inactive."*

6) R. Harrison (ed) Purification Process Engineering, Marcel Dekker, New York (1994)

In Chapter 1 (page 7, Introduction), the author (editor) states:

"Careful attention should always be paid to the biochemistry of the process. Often, the process times become longer and different materials are used as the process is scaled up. Both of these factors lead to unwanted biochemical reactions that reduce yield and purity"

And

"Different processing materials (such as column packings, materials of construction, or chemicals used in the process) can give reactions that bind or denature the product or convert it to undesired by-products."

Risks of protein denaturation during purification are cited in various passages, e.g., p44, 52, 128, 130, 136, 137, 147, 152-154, 173, 175, 211)

e.g., p 128: *"This small stabilization free energy is the basis for the marginal stability of the native structure and its susceptibility to denaturation by relatively small environmental perturbations."*

7) S. Stein (ed) Fundamentals of Protein Biotechnology, Marcel Dekker, New York (1990)

Chapters 6 and 7 discuss issues with respect to denaturation during purification.

Chapter 6 ("Isolation of Natural Proteins", by S. Stein), p145:

"At an early stage in the purification project, it is advisable to test the stability of the biological activity. For example, it may be necessary to work in the cold if the protein loses activity at room temperature. The protein may be more stable at certain values of pH. It is particularly interesting to determine of the activity is preserved in the presence of organic solvents, which would then allow the use of reverse-phase high-performance liquid chromatography (HPLC) for purification. If the protein is stable in the presence of sodium dodecyl sulfate (SDS), it may be possible to use preparative gel electrophoresis as a final purification step."

Chapter 7 (F. Khan and V. Rai) also discuss importance of denaturation in protein isolation/purification. A detailed description of factors influencing the separation of leukocyte interferon is presented on pages 161 and 162.

8) S. Wheelwright, Protein Purification, Hanser Publishers, Munich (1991)

Various passages discuss the impact of denaturation. Proper detergent selection is discussed on page 32, the adverse effects of high shear are discussed on page 62, denaturation by organic solvents and salts during purification are discussed on page 91, denaturation of large proteins during reversed phase chromatography is discussed on page 172, and page 186 covers denaturation during affinity chromatography. The value of ultrafiltration and denaturation during crystallization and organic precipitation are discussed on page 101.

B. References related to enzymes and activated carbon

1) Susan Davis* and Richard G. Burns, "Covalent immobilisation of laccase on activated carbon for phenolic effluent treatment", Appl Microbiol Biotechnol (1992) 37:474-479

The authors state (page 476) that "The maximum enzyme activity (9.80 µmol O_2/min per gram carbon) was reached at 15 mg laccase added/g carbon (11.5 mg laccase bound/g carbon) with another peak at 60 mg laccase added/g carbon (41 mg laccase bound/g carbon), showing 82%+/-7% of the maximum activity. As a result, 11.5 mg laccase bound/g carbon was chosen as the level necessary to provide the highest immobilised laccase activity."

This statement indicates that they were only able to retain 82 +/- 7% of the original activity, that is, there was some activity loss on contact with activated carbon.

2) Sofia Sotiropoulou, Nikos A. Chaniotakis, "Lowering the detection limit of the acetylcholinesterase biosensor using a nanoporous carbon matrix", Analytica Chimica Acta 530 (2005) 199–204

The authors noted an improvement in stability due to immobilization, but no change in initial activity.

3) A.S. Rani, M.L.M. Das, S. Satyanarayana) "Preparation and characterization of amyloglucosidase adsorbed on activated charcoal", Journal of Molecular Catalysis B: Enzymatic 10 (2000) 471–476.

The authors immobilized glucoamylase onto activated carbon, and noted that the activity of the immobilized enzyme was 10% lower than the activity of the soluble enzyme. They state:

"After immobilization on charcoal, the activity of AMG was calculated as 3111 units/min/ml (Fig. 1). It is 90% when compared to the activity of native enzyme - 3428 units. The decrease in the activity of the immobilized enzyme when compared to the native enzyme was probably due to change of the enzyme itself, physical and chemical properties of carriers, and steric hindrance caused by the carrier at the active site."

4) Y. K. CHO* and J. E. BAILEY, "Immobilization of Enzymes on Activated Carbon: Properties of Immobilized Glucoamylase, Glucose Oxidase, and Gluconolactonase" Biotechnology and Bioengineering, Vol. XX, Pp. 1651- 1665 (1978)

Cho and Bailey immobilized glucoamylase, glucose oxidase, and gluconolactase onto activated carbon using a carbodiimide coupling procedure. Various activity assays were conducted. They observed (p1664):

"almost 50% of the soluble gluconolactonase activity is recovered by 1 g carbon, while the carbon-glucose oxidase preparation exhibits only 10% of the soluble glucose oxidase activity per gram of carbon".

Contacting these enzymes with activated carbon has thus led to a 50 to 90% loss of activity.

5) Andreas Naundorf, Katsumi Ajisaka, "Purification of a-*N*-acetyl-galactosaminidase from *Aspergillus niger* and its use in the synthesis of GalNAc-a-(13O)-serine", Enzyme and Microbial Technology 25 (1999) 483–488

The authors carry out a multi-step purification procedure, in which column chromatography using activated carbon comprised one of the steps. Enzyme activity was measured at various stages in the process. Approximately 70% of the desired enzyme activity was lost during purification, although the specific activity (units per mg of total protein) increased, due to removal of non-essential proteins.

6) Kibarer, Gunay; Akovali, Guneri, "Optimization studies on the features of an activated charcoal-supported urease system, Biomaterials". Vol. 17, no. 15, pp. 1473-1479. 1996

The authors note that (page 1474) *"Activity measurements by Berthelot reaction have shown that at an initial urea concentration of* 5×10^{-3} *mol* dm^{-3}*, 80% of the enzyme remains active on charcoal..."*

Thus, the article demonstrates another instance where activity was lost on exposure to activated carbon.

7) JAMES E. BAILEY, and Y. K. CHO "Immobilization of Glucoamylase and Glucose Oxidase in Activated Carbon: Effects of Particle Size and Immobilization Conditions on Enzyme Activity and Effectiveness" , Biotechnology and Bioengineering, Vol. XXV, Pp. 1923-1935 (1983)

The article summary states:

> Glucoamylase and glucose oxidase have been immobilized on carbodiimide-treated activated carbon particles of various sizes. Loading data indicate nonuniform distribution of immobilized enzyme within the porous support particles. Catalysts with different enzyme loading and overall activities have been prepared by varying enzyme concentration in the immobilizing solution. Analysis of these results by a new method based entirely upon experimentally observable catalyst properties indicates that intrinsic catalytic activity is reduced by immobilization of both enzymes. Immobilized glucoamylase intrinsic activity decreases with increasing enzyme loading, and similar behavior is suggested by immobilized glucose oxidase data analysis. The overall activity data interpretation method should prove useful in other immobilized enzyme characterization research, especially in situations where the intraparticle distribution of immobilized enzyme is nonuniform and unknown.

Note the authors' clear statement that intrinsic catalytic activity is reduced by immobilization, i.e., after contacting with activated carbon.

C. References Related to Protein Structure and Circular Dichrosim

1) Nathan J. Roth, Robert M. Penner, and Reuben E. Huber, "β-Galactosidases (*Escherichia coli*) with Double Substitutions Show That Tyr-503 Acts Independently of Glu-461 but Cooperatively with Glu-537, Journal of Protein Chemistry, Vol. 22, Nos. 7/8, November 2003

The goal of this study was to create and purify a modified β-galactosidase. CD was used to compare the wild and modified enzymes. They purified the single and double-modified forms to >98% purity, and noted that:

"*gross physical properties such as size, charge, and aggregation were not changed. Of the most significance was the fact that each had the same retention time as wild-type in size-exclusion columns and migrated the same distance on nondenaturing PAGE, indicating that the normal tetrameric structure was intact. In addition, CD spectra of the double-substituted enzymes were essentially identical to the CD spectrum of wild-type β-galactosidase.*"

Thus, the goal of the purification was to show that structural changes had not occurred, and CD was used to confirm this.

2) A. Sadana, Bioseparation of Proteins, Academic Press, San Diego (1998)

Chapters 6 and 7 provide discussion on the use of CD for establishing protein structure and correlating this information to activity. Case 4 (page 187) is particularly useful.

Page 187: "*In CTAB and EO_4C_{12} systems, no conformational changes were observed, but in AOT, conformational changes were significant*"

Page 245 "*A change in the conformation of the enzyme molecule is indicated by a resultant loss of the enzyme's activity.*"

3) P. Majunath, B.C. Shenoy, M.R. Raghavendra Rao, Fungal Glucoamylases, J. Appl. Biochem., vol 5, 235 - 260 (1983)

Review discusses features of glucoamylases from various sources, including activity, structure, purification, multiplicity of forms, substrate specificity, etc.

Page 246 discusses CD, and its use to correlate activity to structural features:

"*After periodate treatment, there is not much immediate change in the helical content or in the near-uv spectra. However, after 10 days of storage at 4°C, there is a considerable change in the near-uv CD and the α-helical content decreased from 18 to 6% with the complete loss of activity.*"

"Gross conformational changes are seen in enzyme subjected to a combination of periodate and heat."

Further discussion on page 250:

"CD spectra of NBS-oxidized enzyme had the same values as the native one between 200 and 240 nm, but the troughs at 267, 277, and 285 nm disappear on oxidation. Thus, the loss of activity and conformation was also associated with the destruction of tryptophan."